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Genome Construction Between Bacterial Species *In Vitro*: Replication and Expression of *Staphylococcus* Plasmid Genes in *Escherichia coli*

(transformation/R plasmid/antibiotic resistance/restriction endonuclease/recombination)

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ABSTRACT **Genes carried by *Eco*RI endonuclease-generated fragments of *Staphylococcus* plasmid DNA have been covalently joined to the *E. coli* antibiotic-resistance plasmid pSC101, and the resulting hybrid molecules have been introduced into *E. coli* by transformation. The newly constructed plasmids replicate as biologically functional units in *E. coli*, and express genetic information carried by both of the parent DNA molecules. In addition, electron microscope heteroduplex analysis of the recombinant plasmids indicate that they contain DNA sequences derived from *E. coli* and *Staphylococcus aureus*. Recombinant molecules can transform other *E. coli* cells for penicillin-resistance markers originally carried by the staphylococcal plasmid, and can be transferred among *E. coli* strains by conjugally proficient transfer plasmids.**

Cohen *et al.* (1) have recently reported that "hybrid"* DNA molecules constructed *in vitro* by the joining of *Eco*RI (2, 3) endonuclease-generated fragments of separate plasmids can form new biologically functional replicons when inserted into *Escherichia coli* by transformation (4). Plasmid DNA species isolated from such transformed cells possess genetic properties and nucleotide base sequences from both of the parent DNA molecules.

The previously reported method for obtaining biologically functional hybrid DNA species having segments derived from diverse sources appears to be potentially applicable for the introduction of genetic material from various prokaryotic or eukaryotic organisms into *E. coli;* the antibiotic-resistance plasmid used in earlier experiments (i.e., pSC101) (1, 5) is especially useful for the selection of hybrid plasmids constructed *in vitro* from *Eco*RI-generated DNA fragments, since its replication machinery and its tetracycline-resistance gene(s) remain intact after its cleavage by the endonuclease (1).

The present report describes transformation of *E. coli* by hybrid plasmid molecules that have been constructed *in vitro* from *Eco*RI endonuclease-generated fragments of unrelated plasmid DNA species isolated from *E. coli* and *Staphylococcus aureus*. Such hybrid plasmids, which replicate autonomously in *E. coli* and which can be transferred to other bacterial cells by conjugally proficient *E. coli* plasmids, have been shown to carry DNA nucleotide sequences from *E. coli* and *Staphylococcus* and to express genetic information derived from both bacterial species.

* "Plasmid chimeras" might be more appropriate here, but we have not been able to establish a consensus among our colleagues for a definite terminology.

MATERIALS AND METHODS

Staphylococcus aureus strain 8325 containing the plasmid pI258 (6–8), which expresses resistance to penicillin, erythromycin, cadmium, and mercury, was obtained from R. Novick. A mutant of *E. coli* strain C600, defective in restriction and modification functions (C600 $r_K^-m_K^-$) (9), was used for initial selection of *E. coli–Staphylococcus* hybrid plasmids. Other bacterial strains, the tetracycline-resistance plasmid pSC101, and the procedures used (1, 4, 5, 10, 11, 21) for isolation of covalently-closed circular plasmid DNA from *E. coli*, transformation of *E. coli* by plasmid DNA, electron microscopy, plasmid heteroduplex studies, and agarose gel electrophoresis of *Eco*RI endonuclease-generated fragments have been described previously. The conditions used for growth of *Staphylococcus aureus*, and for isolation of staphylococcal plasmid DNA were described by Lindberg *et al.* (12). *Eco*RI restriction endonuclease and *E. coli* DNA ligase were gifts of H. Boyer or P. Modrich and I. R. Lehman, respectively, and were used as described (3, 13, 19), as indicated in Table 1.

RESULTS

Covalently-closed circular staphyloccal DNA, isolated by CsCl–ethidium bromide gradients, was cleaved by *Eco*RI endonuclease, and was examined by agarose gel electrophoresis (Fig. 1) and analytical CsCl gradient centrifugation (Fig. 2). As seen in Fig. 1*a*, the staphylococcal plasmid pI258 is cleaved into four separate fragments having calculated molecular weights of 7.9, 4.6, 4.2, and 1.4×10^6. The sum of the molecular weights of these fragments (18.1×10^6) is in general agreement with the molecular weight reported previously for this plasmid (7, 8), and with the molecular weight calculated (14) from contour-length measurements (about 9.5 μm) of the intact molecule that we have obtained by electron microscopy (Fig. 4, and unpublished data).

The *E. coli* tetracycline-resistance plasmid, pSC101 (1, 5), was used for selection of *E. coli–Staphylococcus* hybrid plasmids. As reported (1), cleavage of pSC101 DNA by the *Eco*RI endonuclease occurs at one site, resulting in formation of a single linear fragment (Fig. 1*b*) having a molecular weight of 5.8×10^6 and a buoyant density in CsCl of 1.710 g/cm^3.

Heterogeneity of base composition of the various component *Eco*RI-generated fragments of pI258 is evident from the buoyant density data shown in Fig. 2. In addition to a main peak banding at a buoyant density of 1.691 g/cm^3, which is nearly identical to the buoyant density of *Staphylococcus* chromosomal DNA (15) and of the untreated plasmid molecule, the

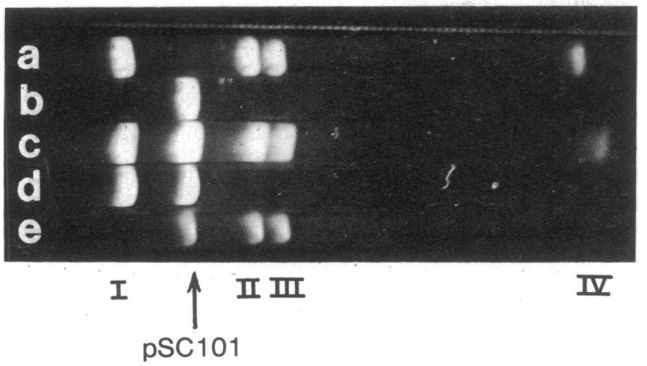

FIG. 1. Agarose gel electrophoresis (21) of *Eco*RI digests of plasmid DNA. The procedure of Helling, Goodman, and Boyer (in preparation) was used, as described (1). Samples were subjected to electrophoresis for 19 hr. The molecular weight of each fragment in the range of 1 to 200 × 10^5 was determined from its mobility in the gel relative to the mobilities of DNA standards of known molecular weight. (*a*) pI258. The molecular weights calculated for the four fragments derived from the pI258 plasmid are (from *left* to *right* as identified in figure) *I*, 7.9; *II*, 4.6; *III*, 4.2; and *IV*, 1.4. All molecular weights have been multiplied by 10^{-6} and may be assumed to have a precision of ±10%. (*b*) pSC101. Molecular weight equals 5.8 × 10^6 (1, 5). (*c*) pSC101 plus pI258. (*d*) pSC112. (*e*) pSC113. The molecular weights of the intact hybrid plasmids estimated from the weights of the component fragments are: pSC112, 13.7 × 10^6; pSC113, 14.6 × 10^6.

*Eco*RI-cleaved plasmid contains at least one DNA fragment, which bands at a buoyant density of 1.699 g/cm³.

Transformation of *E. coli* C600 $r_K^-m_K^-$ was carried out with covalently-closed circular or *Eco*RI-cleaved pSC101 and pI258 plasmid DNA, or a ligated mixture of the two *Eco*RI-treated DNA species (Table 1). As reported (1, 5), tetracycline-resistant transformants were observed after transformation with either untreated pSC101 plasmid DNA or with the endonuclease-cleaved plasmid. No *E. coli* transformants for penicillin resistance or for other resistance markers carried by the *Staphylococcus* plasmid were observed when either covalently-closed-circular pI258 plasmid DNA or an *Eco*RI digest of this plasmid was used alone in the transformation assay. However, penicillin-resistant colonies and colonies that are jointly resistant to both penicillin and tetracycline were obtained after transformation with a ligated mixture of *Eco*RI-cleaved pSC101 and pI258 DNA (Table 1). A single clone of each type of transformant was selected for further study.

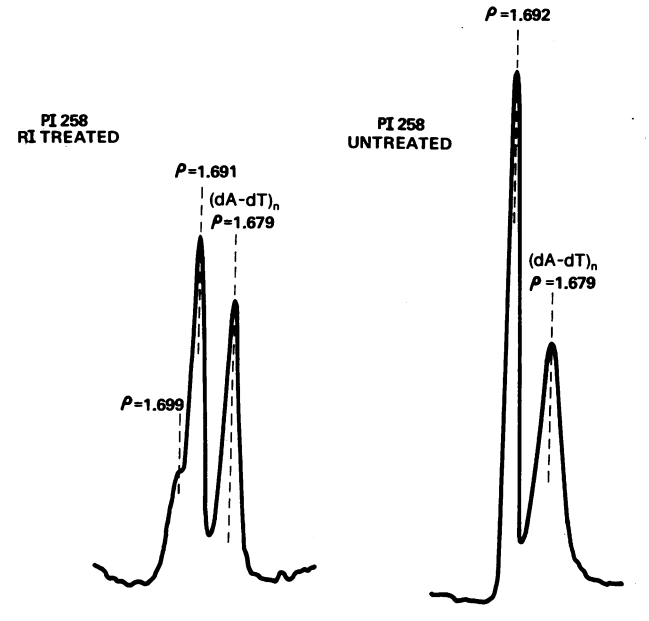

FIG. 2. Analytical ultracentrifugation (Spinco) of *Staphylococcus* plasmid DNA cleaved by *Eco*RI endonuclease (*left*) as described in Table 1, or untreated (*right*). Centrifugation in CsCl (ρ = 1.690 g/cm³) (10) was carried out for 26 hr at 44,000 rpm in the presence of a $(dA\text{-}dT)_n$ (ρ = 1.679 g/cm³) density marker. Densitometer (Gilford) tracings of photographs taken during centrifugation are shown.

Covalently-closed circular DNA isolated from each of the selected clones was purified as described (4, 5, 10), and was examined by centrifugation in analytical CaCl gradients. As seen in Fig. 3, the plasmid DNA isolated from an *E. coli* transformant carrying resistance to both penicillin and tetracycline (plasmid designation pSC112) banded at a buoyant density of 1.700 g/cm³, while the plasmid DNA obtained from the clone expressing only penicillin resistance (plasmid designation pSC113) had a buoyant density of 1.703 g/cm³ in CsCl.

Treatment of both plasmid DNA species with *Eco*RI enzyme and subsequent CsCl gradient analysis of the fragments (Fig. 3) indicated that pSC112 contains fragments having two different buoyant densities: a DNA species banding at 1.710 g/cm³, which is identical to the buoyant density of

TABLE 1. *Transformation of C600 $r_K^-m_K^-$ by pSC101 and pI258 plasmid DNA*

DNA	Transformants/μg DNA	
	Tc	Pc
pSC101 closed circular	1 × 10^6	<3
pI258 closed circular	<3.6	<3.6
pSC101 + pI258 untreated	9.1 × 10^5	<5
pSC101 + pI258 *Eco*RI-treated	4.7 × 10^3	10

Transformation of *E. coli* strain C600 $r_K^-m_K^-$ with covalently-closed circular pSC101 (0.83 μg/ml) or pI258 (7.4 μg/ml) DNA and selection of transformants resistant to tetracycline (*Tc*, 25 μg/ml) or penicillin (*Pc*, 250 U/ml) were carried out as described (4). Covalently-closed circular pSC101 and pI258 plasmid DNA, isolated as indicated in *Methods*, were separately cleaved by incubation 37° for 15 min in 0.2-ml reaction mixtures containing DNA (40 μg/ml), 100 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM NaCl, and excess (2 units) *Eco*RI endonuclease (3) in 1-μl volume. After an additional incubation at 60° for 5 min to inactivate the endonuclease, aliquots of the two cleaved species were mixed in a ratio of 3 μg of pI258: 1 μg of pSC101 and annealed at 2–4° for 48 hr. Subsequent ligation was carried out for 6 hr at 14° (19) in 0.2-ml reaction mixtures containing 5 mM $MgCl_2$, 0.1 mM NAD, 100 μg/ml of bovine-serum albumin, 10 mM $(NH_4)_2SO_4$ (pH 7.0), and 18 U/ml of DNA ligase (13). Ligated mixtures were incubated at 37° for 5 min and chilled in ice water. Aliquots containing 3.3–6.5 μg/ml of total DNA were used directly in the transformation assay (4). Transformation frequency is expressed in terms of transformants per μg of plasmid DNA. No penicillin-resistant transformants were observed when an unligated mixture of *Eco*RI-cleaved pSC101 and pI258 DNA was used. Resistance of *E. coli* transformants to erythromycin, $CaCl_2$, or $HgCl_2$ was not observed at levels expressed by *Staphylococcus aureus* carrying the plasmid pI258.

pSC101 DNA (1, 5), and a second species having a buoyant density (ρ = 1.691 g/cm^3) approximately equal to that observed for the parent *Staphylococcus* plasmid (Fig. 2). pSC113 is composed of DNA fragments having three different buoyant densities in CsCl: at least two separate *Eco*RI-generated fragments of pI258 having buoyant densities of 1.691 and 1.699 g/cm^3 appeared to be contained in the pSC113 hybrid plasmid, in addition to a fragment representing pSC101.

These interpretations were confirmed by electrophoresis of *Eco*RI digests of pSC112 and pSC113 DNA in agarose gels (Fig. 1*d* and *e*). Furthermore, these electrophoresis results indicate that the fragment of pI258 present in the pSC112 *E. coli–Staphylococcus* hybrid plasmid (i.e., fragment I) is absent in pSC113, while the latter plasmid contains fragments II and III of the parent staphylococcal plasmid. Since both pSC112 and pSC113 express penicillin resistance, we conclude that the *Staphylococcus* plasmid pI258 carries at least two genes capable of coding for penicillin resistance. It has not yet been determined whether these genes are duplicates.

Densitometer tracings of photographs taken during CsCl buoyant density gradient centrifugation of *Eco*RI-treated pSC113 DNA indicate that the peak banding at 1.699 g/cm^3 contains less DNA than the peak at 1.691 g/cm^3, and is probably fragment III (molecular weight 4.2 × 10^6).

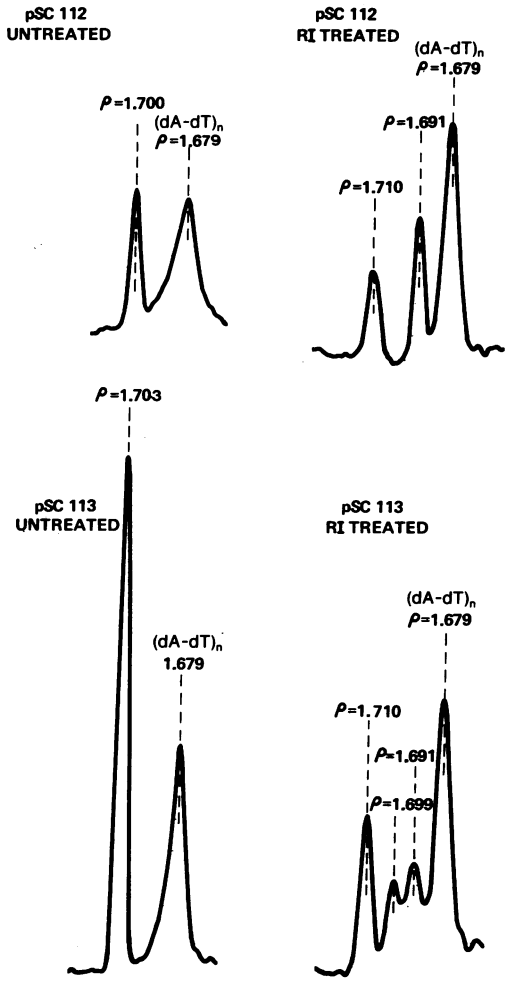

FIG. 3. Analytical ultracentrifugation of DNA comprising pSC112 (*top*) and pSC113 (*bottom*). Cleavage by the *Eco*RI endonuclease (*right*) was carried out as described in Table 1. Centrifugation conditions were as indicated in legend of Fig. 2.

The 7.9 × 10^6-dalton fragment of pI258 included in pSC112 necessarily carries one of the two penicillin-resistance genes identified on the *Staphylococcus* plasmid DNA. It is not yet clear which of the two *Staphylococcus* DNA fragments included in pSC113 contains the other penicillin-resistance gene. It is notable that tetracycline resistance at 25 μg/ml is expressed *only* by pSC112, although an *Eco*RI fragment comprising the tetracycline-resistance plasmid pSC101 is also included in pSC113 (Fig. 1), which failed to express tetracycline resistance at the same level as the parent plasmid. However, a tetracycline-resistance plasmid having physical and biological properties indistinguishable from those of pSC101 could be recovered after transformation of *E. coli* by an *Eco*RI digest of pSC113 DNA (Chang and Cohen, unpublished data). Although the mechanism by which expression of tetracycline resistance is reduced in pSC113 is presently unclear, nonexpression of plasmid-borne antibiotic resistance has been reported to occur by DNA insertion (5, 11).

Transformation of *E. coli* C600 and of a restriction-minus, modification-minus mutant of this strain by pSC112 or pSC113 plasmid DNA is shown in Table 2*A*. As seen in this table, both of these *E. coli–Staphylococcus* plasmid species are capable of transforming *E. coli* to penicillin resistance. In addition, the hybrid plasmids pSC112 and pSC113 can be mobilized to a *restriction-competent E. coli* strain (CR34) by the conjugally proficient transfer plasmid I. (Table 2*B*). The frequency of transformation to the $r_K^+m_K^+$ strain was about 50-fold lower than the frequency observed with C600

TABLE 2. *Transformation and transfer of* E. coli–Staphylococcus *recombinant plasmids*

(*A*) *Transformation* (transformants/μg DNA)

	C600 $r_K^-m_K^-$		C600 $r_K^+m_K^+$	
Plasmid DNA	Pc	Tc	Pc	Tc
PSC112				
Untreated	170	180	13	34
RI treatment	7.5	170	7.5	16
PSC113				
Untreated	13,000	11	160	2.5
RI treatment	510	170	5.2	2.5

(*B*) *Conjugal transfer*

	Frequency of transfer of antibiotic resistance	
	Pc	Tc
SP(I) × C600 $r^-_Km^-_K$ (pSC112) × CR34N	4 × 10^{-9}	4 × 10^{-9}
SP(I) × C600 $r^-_Km^-_K$ (pSC113) × CR34N	7 × 10^{-5}	<2 × 10^{-9}

(*A*) Transformation. Covalently-closed circular, *Eco*RI-cleaved pSC112 or pSC113 plasmid DNA was used to transform (4) either *E. coli* C600 $r_K^+m_K^+$ or C600 $r_K^-m_K^-$ for resistance to tetracycline or penicillin, as indicated in Table 1 and ref. 4.

(*B*) Conjugal transfer. pSC112 and pSC113 were mobilized by *Salmonella panama* SP (I) (16, 17) to a nalidixic acid-resistant mutant of CR34, by a modification of the procedure of Anderson and Lewis (18), Selection of the final recipient was carried out with nalidixic acid (100 μg/ml) and penicillin (250 U/ml) or tetracycline (25 μg/ml). Transfer frequency is expressed as antibiotic-resistant colonies per total number of viable cells.

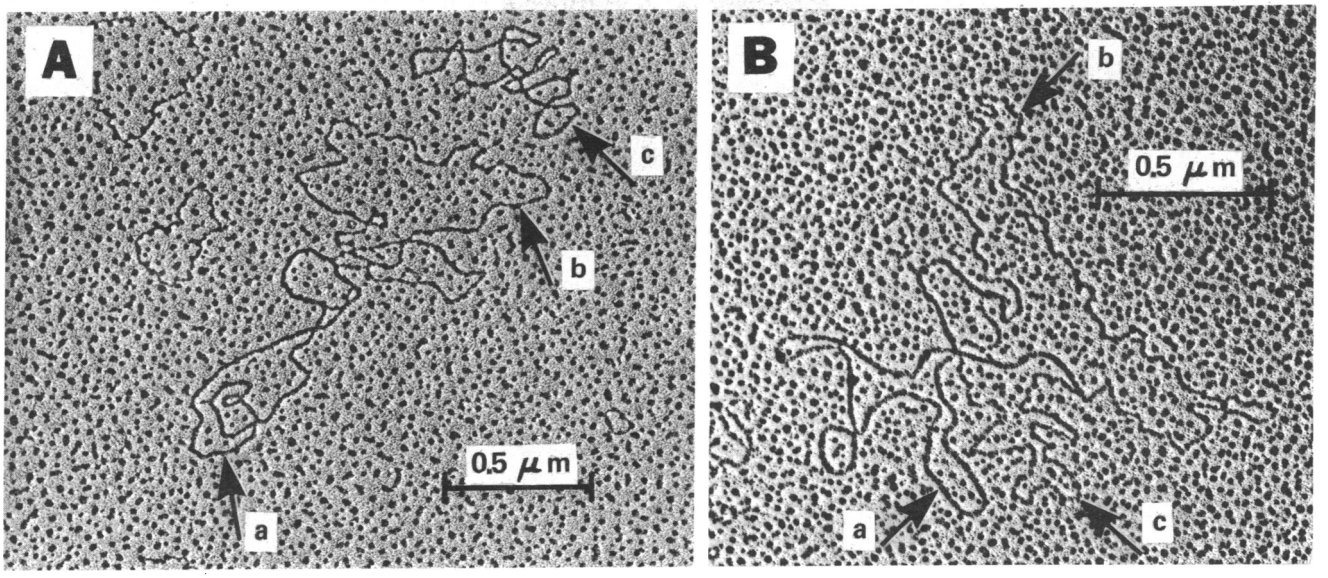

FIG. 4. (*A*) Heteroduplex of pSC101/pSC112. The region of homology is indicated by (*a*). The single-stranded loop (*b*) contains the DNA contribution of pI258 to the pSC112 hybrid plasmid. (*c*) Double-stranded molecule of pSC101 serving as internal standard for contour-length measurements. (*B*) Heteroduplex of pI258/pSC112. (*a*) Double-stranded region of homology (i.e., the segment of both plasmids containing fragment I of pI258). (*b*) Single-stranded region containing the *Eco*RI fragments of pI258 that are absent in pSC112. (*c*) Single-stranded region containing the DNA contribution of pSC101 to pSC112.

$r_K^- m_K^-$, suggesting that the staphylococcal DNA segments included in each of these plasmids contain sites susceptible to attack by the K restriction enzyme of *E. coli* (9).

Table 2 also shows that although pSC113 did not express tetracycline resistance in the clone of its origin, it is expressed in cells *transformed* by pSC113 plasmid DNA. A parallel finding was reported previously (5) for the tetracycline resistance marker carried by the R6-5 plasmid.

Heteroduplexes between pSC112 and each of its parent plasmids are shown in Fig. 4. As seen in this figure, the heteroduplex pSC101/pSC112 shows a double-stranded region approximately 3 μm in length and a much longer single-stranded region which represents the contribution of pI258 to the recombinant *E. coli–Staphylococcus* plasmid. The heteroduplex formed between pI258 and pSC112, also shows both a duplex region and two regions of nonhomology (substitution loops), which contain: (*1*) the DNA contribution of pSC101 to pSC112 and (*2*) the three *Eco*RI-generated fragments of pI258 that are absent in the hybrid plasmid.

DISCUSSION AND SUMMARY

The results of these experiments indicate that *Staphylococcus* plasmid genes can be linked to *E. coli* plasmid DNA by ligation of overlapping single-stranded ends (19, 20) of *Eco*RI endonuclease-generated fragments of both plasmids. The resulting hybrid molecules can be introduced by transformation into *E. coli* where they replicate as biologically functional units. The recombinant plasmids contain DNA nucleotide sequences derived from both *E. coli* and *Staphylococcus*, as demonstrated by electron microscope heteroduplex analysis. Moreover, genetic information carried by the *Staphylococcus* DNA is expressed in *E. coli*.

Earlier investigations (1) demonstrated that new, biologically functional plasmid species can be constructed *in vitro* with fragments of larger *E. coli* plasmids. The replication and expression of genes in *E. coli* that have been derived from a totally unrelated bacterial species (i.e., *Staphylococcus aureus*) now suggest that interspecies genetic recombination may be generally attainable. Thus, it may be practical to introduce into *E. coli* genes specifying metabolic or synthetic functions (e.g., photosynthesis, antibiotic production) indigenous to other biological classes. In addition, these results support the earlier view that antibiotic-resistance plasmid replicons such as pSC101 may be of great potential usefulness for the introduction of DNA derived from eukaryotic organisms into *E. coli*, thus enabling the application of bacterial genetic and biochemical techniques to the study of eukaryotic genes.

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